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# Impact of season and rearing site on the physiological and immunological parameters of the Manila clam *Venerupis* (= *Tapes*, = *Ruditapes*) *philippinarum*

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## Abstract

Juvenile clams were distributed in four rearing sites selected for their varied ecological characteristics to assess the effects of environmental conditions on the physiological and immunological parameters, and Brown Ring Disease (BRD) status. Clams were sampled every 3 months for 15 months at each site. Brittany rearing sites, especially the Bay of Brest, showed the worst performances in terms of immunological and physiological indices and disease status, while the best were obtained in Marennes ponds. When the health of the clams was compared to assess seasonal effects, the winter clearly was a stressful period. A combination of bad rearing site and winter conditions led to major mortalities in the Bay of Brest in February. In other sites, winter mortalities were low. Condition index, total haemocyte count and haemocyte size were greatly affected by seasonal variation whereas haemocyte complexity and lysozyme content were more affected by the location of the site. Growth and haemolymph protein content were affected by both season and sites. Linear regressions between and within the physiological and immunological parameters indicated that large haemocyte size was related to low total haemocyte count (THC) and low haemocyte mortality. This relationship suggests a reduction in the cell division rate. Total haemocyte count and protein and lysozyme concentrations were positively correlated to the condition index. Seeded clams showed very low BRD prevalence in all sites and for all seasons; however, high prevalence was observed in natural stocks from one of the studied sites (Gulf of Morbihan), suggesting that hatchery-seeded clams may be more resistant to BRD and may be worthy of further studies.

**Keywords:** Shellfish aquaculture; Clams; Immunology; Physiology; Disease

## 1. Introduction

Environmental factors such as temperature, salinity, dissolved oxygen, nutrients, toxicants, and parasites, that exhibit intermittent and short-term fluctuations, affect health of cultivated or exploited marine bivalves. Cellular defence-related parameters are known to be especially sensitive to the variation of these factors and the physiological and health status of bivalves tested (Chu, 2000).

In Europe, the Manila clam *Venerupis philippinarum*, which is of Indo-Pacific origin, was introduced for aquaculture purposes to France between 1972 and 1975 and later to England, Spain and Italy (Flassch and Leborgne, 1992). In France, this new venerid culture became increasingly widespread until 1986, demonstrating its adaptation to the local environmental conditions along the Atlantic coast. *V. philippinarum* is, however, often affected by high mortality in late winter which considerably limited further expansion of this venerid culture. This mortality has been associated with low condition index, and low glycogen and lipid contents during this season (Goulletquer, 1989) and with Brown Ring Disease (BRD), a bacterial disease caused by *Vibrio tapetis* (Paillard et al., 1989; Paillard and Maes, 1990). Environmental factors, such as freezing winter temperatures (Bower, 1992), and turbidity (Goulletquer, 1989) have been suggested as causing this seasonal mortality. Low temperature was also demonstrated to favour BRD prevalence which is facilitated by immune-compromised conditions of the clams (Paillard et al., 1997). However, how environmental conditions affect defence mechanisms of Manila clams is unknown. Defence mechanisms in other bivalves, namely oysters and mussels, have been shown to be affected by environmental factors (Fisher and Newell, 1986; Chu and La Peyre, 1989; Santare'm et al., 1994; Chu et al., 1995; Fisher et al., 1996, 2000; Carballal et al., 1998; Volety et al., 1999; Oliver et al., 2001). Establishment of correlation between environmental parameters and immunological and disease status is difficult, especially in field studies; however, collected information can be valuable in understanding environment–host–pathogen interactions and in evaluating quality of potential culture sites.

The present field experiment was designed to investigate influences of environmental conditions of various rearing sites and season on physiological and immunological parameters, and on BRD development in the clam *V. philippinarum*. Flow cytometry methods were applied to determine total haemocyte counts, haemocyte viability and morphological characteristics. Lysozyme and protein contents were analysed because they have been shown to be sensitive to environmental factors in other bivalves (Fisher and Newell, 1986; Chu and La Peyre, 1989; Fisher et al., 1996; Ordas et al., 2000). The study also explored whether correlations between some environmental factors and physiological and immunological parameters can be established.

## 2. Material and methods

The experimental juvenile Manila clams (*V. philippinarum*), originated from a single stock (same spat origin, about 5 months old, 1–2 g) provided by a private farm (SATMAR, Marennes, France). From this stock, 8000 individuals were distributed in April 2000 to four rearing sites located along the French coast from north to south (Fig. 1) that were selected for their various ecological characteristics (climate, hydrodynamics). Sites can be classified in three ecosystem types: Chausey Islands, oceanic; Bay of Brest and Gulf of Morbihan, estuarine; Marennes, pond. All sites are used for aquaculture purpose and potentially affected by Brown Ring Disease (BRD). The sediment in Chausey consisted of a mixture of sand and mud in equivalent proportions whereas the sediment in the three other sites is mostly composed of mud.

The deploying of the clams was performed in two different manners, depending on the rearing sites. In the Bay of Brest, the Gulf of Morbihan and Marennes, the juvenile clams were seeded manually. In Chausey Islands, the clams were seeded mechanically. The experimental sites were checked monthly for any damage. The temperature profiles were obtained with in situ probes over the experiment at each site.

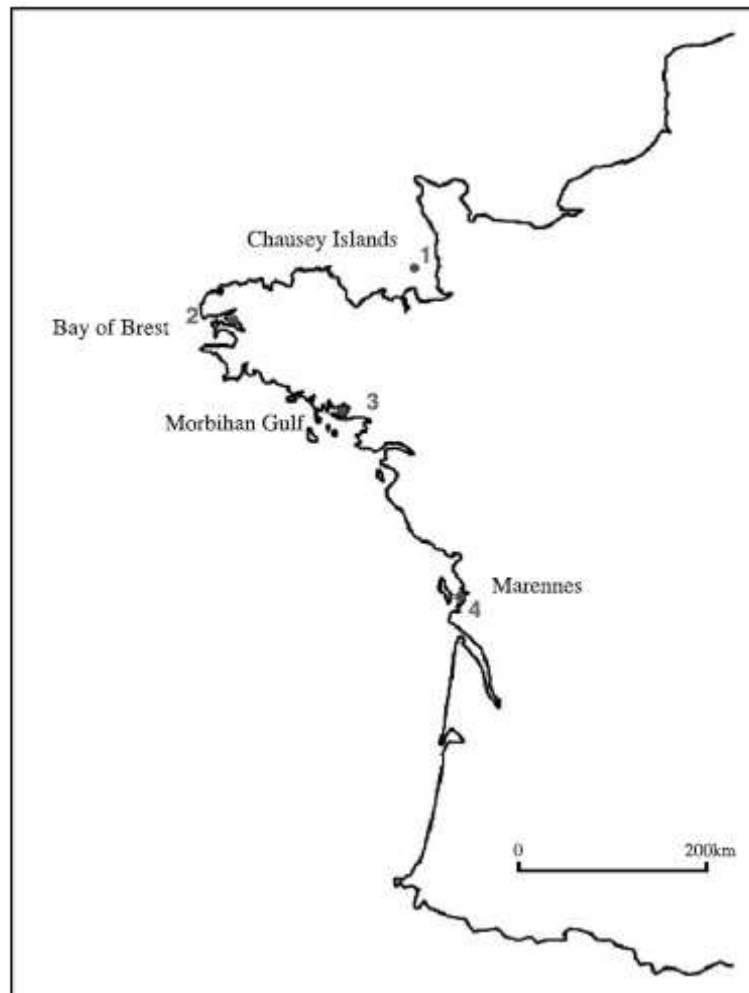


Fig. 1. Location in France of the different rearing sites utilised for the experiment.

Samples of 100 clams were collected every 3 months for 15 months at each site: in July and October 2000, February, April and August 2001. The clams were harvested and shipped in a refrigerated box the first day and rapidly processed the following day in the laboratory. Disease progression was monitored according to Paillard

and Maes (1994) with all 100 clams from each site observed for macroscopic and microscopic indications of BRD disease. Twenty individual clams, randomly selected among the 100 sampled clams, were used for physiological indices. For each individual clam, the total weight (shell + meat) was measured immediately upon arrival. After dissection, the wet weight of the meat was then measured. After incubation for 48 h in an oven at 60 °C, the dry weight of the meat and the shell were measured separately. Condition index (CI) was then calculated as  $CI = (\text{dry weight meat} / \text{dry weight shell}) \times 100$  according to Mann and Glomb (1978). Length measurement corresponds to the maximal antero-posterior length (mm).

Twenty other individual clams, randomly selected among the 100 sampled clams, were used for immunological analysis. Haemolymph was collected according to Oubella et al. (1993) from the posterior adductor muscle sinus with a 1-ml hypodermic syringe (25 G needle) through the shell hinge. This procedure is considered as nondestructive since even repeated haemolymph sampling from the adductor muscle had no measurable effect on survival of the clam *V. philippinarum* (Paillard and Ford, 2002). Except for samples of July 2000, all the analyses were performed on individual clams. Because of the small size of clams sampled in July 2000, large enough haemolymph samples could not be collected to run individual immunological analyses. Haemolymph pools (3 pools of 10 animals) were thus constituted to run the immunological analysis. After collection of haemolymph, 200  $\mu$ L of fluid was immediately mixed with an anti-aggregant solution for bivalve haemocyte (AASH), prepared according to Auffret and Oubella (1995), maintained on ice until flow cytometry analysis of the total haemocyte count (THC), the percentage of haemocyte mortality, and the cell size and complexity. The remainder of the haemolymph sample was subdivided in (1) 150  $\mu$ L for lysozyme assay, and (2) 5  $\mu$ L for haemolymph total protein content. Samples for lysozyme and total protein content were frozen at -80 °C until analysis.

Flow cytometry methods were applied to determine total haemocyte count, haemocyte cyto-morphology and percentage of dead haemocytes, and were expected to considerably ease measurement of these parameters which are time-consuming and technically demanding by microscopic methods. Total haemocyte count (THC) was assessed by counting at least 10000 particles and by recording the elapsed time. The flow rate of the flow cytometer was controlled regularly and varied little under our working conditions throughout the year. THC was then calculated according the following formula  $THC = 10000 \times \text{elapsed time} / \text{measured flow rate}$ . Results were expressed as cells  $\text{mL}^{-1}$ . Forward scattering (FSC) and side scattering (SSC) parameters which are, respectively, related to cell size and cell complexity, were also recorded on total cells. All the samples were analysed using previously determined instrument settings, and because of this, the haemocyte size (FSC value) and complexity (SSC value) were expressed as Arbitrary Units to allow site and season comparisons.

In order to measure haemocyte mortality, the haemocyte suspensions were labelled with propidium iodide to a final concentration of 10  $\mu\text{g mL}^{-1}$ , 10 min prior the flow cytometry analysis. Dead cells that lost membrane integrity incorporated propidium iodide by DNA intercalation and the resulting fluorescence was measured on the FL2 fluorescent detector. Results were presented by giving the percentage of dead cells present in samples. Protein analysis was carried out in triplicate on 5- $\mu$ L samples using the Bio-Rad DC Protein Assay, based on the protein determination method of Lowry et al. (1951). The protein determination was made as described by the manufacturer. Briefly, 25  $\mu$ L of solution A was added to the sample and mixed for 1 min. Then, 200  $\mu$ L of solution B was added and also mixed for 1 min. After 15 min of incubation, optical density was measured with a micro-plate reader at 620 nm. A standard curve was developed from the standard protein stock solution provided by the manufacturer. Results were expressed as mg of protein/mL.

Lysozyme concentration in whole haemolymph samples was measured after samples had been thawed. This was done in triplicate for each sample, and compared against hen egg-white lysozyme standards (0.6–40  $\mu\text{g mL}^{-1}$ ) using a 96-well micro-plate method. Twenty microliters of sample was dispensed per well and 180  $\mu$ L of *Micrococcus leikodektus*, suspended in 0.06 M sodium phosphate buffer (pH 6.4) to an  $OD_{600} = 0.4$ , added to each. To permit more accurate measurement of the low levels of lysozyme in these samples, the plates were incubated for 1 h at room temperature before reading at 540 nm. Data were processed using nonparametric method (Kruskal–Wallis test). THC values were log transformed and percentage of haemocyte mortality values were transformed (arcsin of the square root) prior to statistical analysis. Data are presented in figures as untransformed. Differences were considered to be statistically significant at  $p < 0.05$  and correlation were retained when  $p < 0.1$ .

### 3. Results

Seasonal variations of temperature within sites were greater than those between sites (Fig. 2). In summer, the temperature was higher in the sites located in the south of France (Marennes) than those in the north. The highest temperature (24°C) was observed in Marennes and the lowest (8°C) in Chausey and Gulf of Morbihan. The differences in monthly mean temperature between sites were higher during summer than during winter.

The experimental juvenile Manila clams were disease free prior to deployment in April 2000. Also, 500 additional juvenile clams maintained for 1 month in a laboratory aquarium without food supplementation (stressful conditions) remained BRD-free thus confirming that the seeded animals were BRD-free. The clams

deployed back into the site of origin (Marennnes), stayed disease free for the 15 months of the field study. The clams planted in other sites developed a very low prevalence of BRD, which reached a maximum of 3.5% in the Gulf of Morbihan and the Bay of Brest (Fig. 3).

The BRD was also monitored in a natural population of 1–3-year-old clams from the Gulf of Morbihan, near the experimental clams, and revealed much higher BRD prevalence in the natural population: 30% in January and April 2001 and 16% in August 2001 (data not shown). Though BRD was present in natural stocks of the Gulf of Morbihan, experimental clams developed very low BRD during the monitored period. Very high mortalities occurred in the Bay of Brest during the winter (February) and in the Gulf of Morbihan during the second summer. At the time of the observation, all the experimental clams (2000 individuals) were found as empty shells.

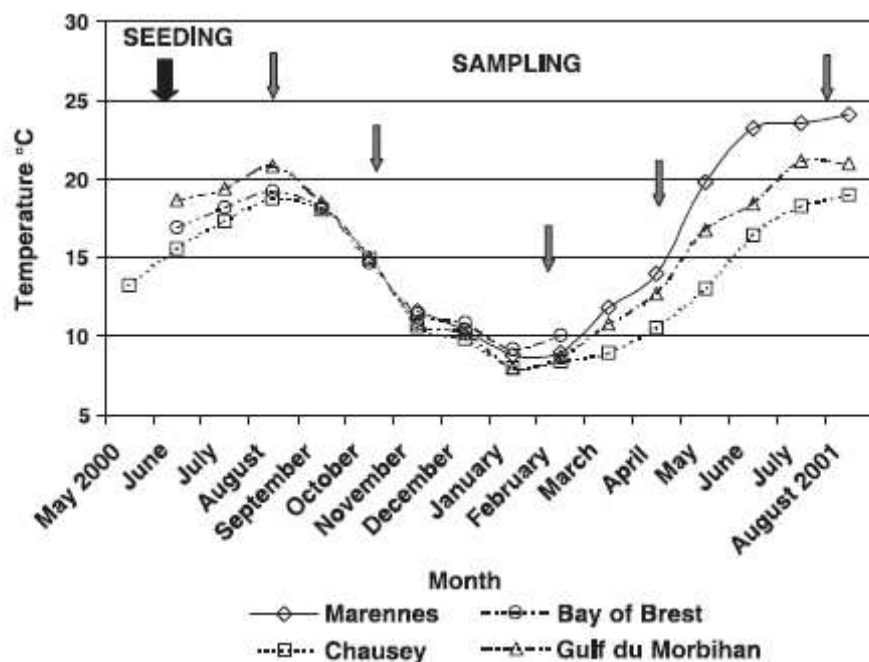


Fig. 2. Monthly mean temperature profile of the rearing sites.

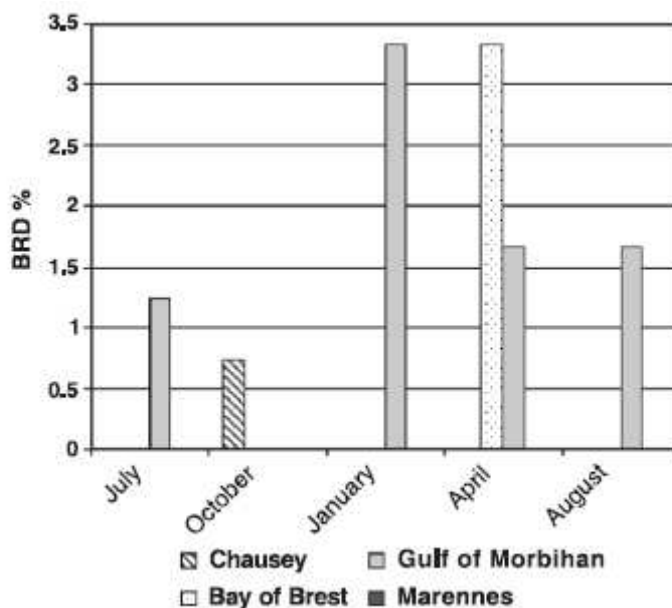


Fig. 3. Brown Ring Disease (BRD) prevalence (clams seeded in April 2000 were disease-free).



Table 1 : Seasonal effects on immunological and physiological parameters

	July		October		February		April		August		P value
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	
Physiological parameters	(n=60)		(n=82)		(n=80)		(n=40)		(n=60)		
Length (mm)	n.d.		35.25	0.48 a	34.44	0.41 a	38.27	0.55 b	41.26	0.69 c	<0.0001
Meat dry weight (g)	0.19	0.01 a	0.35	0.02 b	0.25	0.02 ab	0.53	0.04 c	0.78	0.06 d	<0.0001
Condition index	11.08	0.27 a	6.91	0.17 c	5.06	0.12 d	7.60	0.28 bc	8.50	0.35 b	<0.0001
Hematological parameters	(n=12 pools)		(n=66)		(n=81)		(n=59)		(n=59)		
Haemocyte size (AU)	468.7	9.0 *	569.2	3.3 a	551.8	5.4 b	479.9	7.3 c	438.9	3.0 d	<0.0001
Haemocyte complexity (AU)	439.9	8.8 *	438.3	8.2 a	436.3	5.8 a	384.7	5.6 C	415.5	4.4 b	<0.0001
THC ( $\times 10^6$ cell $\text{ml}^{-1}$ )	n.d.		0.27	0.18 a	0.42	0.04 a	1.35	0.11 c	0.91	0.06 b	<0.0001
Haemocyte mortality (% of total haemocytes)	13.0	1.5 *	6.8	0.7 a	3.0	0.3 b	2.4	0.3 b	2.1	0.2 b	<0.0001
Biochemical parameters			(n=28)		(n=75)		(n=60)		(n=55)		
Lysozyme ( $\mu\text{g ml}^{-1}$ )	n.d.		6.97	0.42 a	4.69	0.36 b	5.20	0.39 b	5.58	0.39 b	<0.001
Protein content ( $\text{mg ml}^{-1}$ )	n.d.		0.98	0.07 a	0.48	0.03 b	0.62	0.03 c	1.00	0.07 a	<0.0001

Results were expressed as mean and standard error (S.E.). Lettering indicates the significant differences between groups of each parameter such that similar letters signify no significant difference between other values of the same parameter. Statistical analysis was performed using Kruskal–Wallis test (K–W).

\* Since hematological data were obtained from pooled hemolymph, they were not included in the statistical analysis testing the seasonal effect on individually sampled hemolymph from October 2000 till August 2001.  
n.d.: not determined.

Length and dry weight (meat) showed a seasonal pattern with marked increases during spring and summer and slight decreases or maintenances of these parameters during the winter (Table 1). The dry weight of clams was significantly affected by the location of the rearing site (Table 2). Clams from Marennes in July were about half the weight of clams from the three other sites ( $P < 0.0001$ ). In contrast, the clams from October collections in Marennes were at least twice the size of those from the other sites ( $P < 0.0001$ ). Though a slight decrease of dry weight occurred during the winter, clams from Marennes remained about twice the weight of those of the other sites ( $P < 0.0001$ ). At the end of the experiment, rearing sites could be classified in the following descending order according to the clam dry weight: Marennes, Chausey and Gulf of Morbihan with final dry weights of 1.24, 0.84 and 0.26 g, respectively. Though to a lesser extent, similar variations between sites were noted for the length, which followed a similar order for sites as the dry weight. The condition index (Tables 1 and 2) of the clams showed a seasonal pattern for all sites. The highest values of CI were observed in summer (11.1 for July 2000 and 8.5 for August 2001) and decreased in autumn (6.9) ( $P < 0.0001$ ). The lowest values were measured during winter (5.0). The seasonal pattern was less marked in Marennes than in other sites. At each sampling date, statistically significant differences of CI were measured between sites ( $P < 0.0001$ ). During autumn and winter, the highest condition index was observed in Marennes. Unlike the weight parameters, the CI of Chausey and Marennes were similar at the end of the experiment (10.5 and 9.7, respectively) but about twice that for the Gulf of Morbihan (5.2).

Total haemocyte count (THC) followed a similar seasonal pattern for all sites with significant differences between sampling dates: the highest THC was observed in spring ( $1.35 \times 10^6$  cells  $\text{ml}^{-1}$ ) and the lowest in autumn ( $0.27 \times 10^6$  cells  $\text{ml}^{-1}$ ) (Table 1,  $P < 0.0001$ ). Sites could also be distinguished statistically according to THC values (Fig. 4). In autumn, clams from Marennes exhibited significant higher THC values ( $0.33 \times 10^6$  cells  $\text{ml}^{-1}$ ) than clams from Gulf of Morbihan ( $0.20 \times 10^6$  cells  $\text{ml}^{-1}$ ) ( $P < 0.01$ ). This pattern changed in winter, the highest THC value was observed in clams from the Chausey Islands whereas the lowest THC value was observed in the clams from Bay of Brest ( $P < 0.001$ ). In the latter site, clams were decimated by mortalities occurring from February to April 2001. Intermediate THC values were measured in Marennes and the Gulf of Morbihan. Similar classification between sites was obtained in spring and summer. In the Gulf of Morbihan, clams exhibited lower THC values ( $0.65$ – $0.96 \times 10^6$  cells  $\text{ml}^{-1}$ ) compared to those of Marennes ( $1.05$ – $1.75 \times 10^6$  cells  $\text{ml}^{-1}$ ) and Chausey Islands ( $1.04$ – $1.37 \times 10^6$  cells  $\text{ml}^{-1}$ ) ( $P < 0.05$ ).

Table 2 : Site effect on physiological parameters during the field survey

	Chausey		Bay of Brest		Gulf of Morbihan		Marennes		P value
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	
July	(n = 15)		(n = 15)		(n = 15)		(n = 15)		
Length (mm)	n.d.		n.d.		n.d.		n.d.		
Meat dry weight (g)	0.22	0.01 b	0.22	0.09 b	0.23	0.02 b	0.11	0.01 a	<0.0001
Condition index	12.84	0.45 b	12.24	0.46 b	9.37	0.29 a	9.86	0.27 a	<0.0001
October	(n = 20)		(n = 21)		(n = 21)		(n = 20)		
Length (mm)	34.45	0.36 b	33.85	0.50 b	31.39	0.47 a	41.59	0.56 c	<0.0001
Meat dry weight (g)	0.27	0.01 b	0.31	0.02 b	0.19	0.01 a	0.64	0.03 c	<0.0001
Condition index	6.03	0.18 a	7.42	0.19 b	5.69	0.17 a	8.55	0.32 c	<0.0001
February	(n = 20)		(n = 20)		(n = 20)		(n = 20)		
Length (mm)	34.34	0.55 b	33.63	0.41 b	31.20	0.51 a	38.58	0.77 c	<0.0001
Meat dry weight (g)	0.21	0.01 a	0.18	0.01 a	0.15	0.01 a	0.44	0.03 b	<0.0001
Condition index	4.83	0.14 a	4.49	0.09 a	4.45	0.17 a	6.47	0.17 b	<0.0001
April	(n = 20)						(n = 20)		
Length (mm)	35.51	0.38 a	dead		n.d.		41.02	0.55 b	<0.0001
Meat dry weight (g)	0.29	0.01 a	dead		n.d.		0.76	0.04 b	<0.0001
Condition index	6.17	0.23 a	dead		n.d.		9.04	0.21 b	<0.0001
August	(n = 20)				(n = 20)		(n = 20)		
Length (mm)	42.38	0.46 b	dead		34.88	0.43 a	46.52	0.62 c	<0.0001
Meat dry weight (g)	0.84	0.04 b	dead		0.26	0.02 a	1.24	0.06 c	<0.0001
Condition index	10.53	0.38 b	dead		5.23	0.18 a	9.74	0.30 b	<0.0001

Results were expressed as mean and standard error (S.E). Lettering indicates the significant differences between groups of each parameter such that similar letters signify no significant difference between other values of the same parameter. Statistical analysis were performed using Kruskal–Wallis test (K–W).

Percentage of dead haemocytes (PDH) ranged from 2% to 13% throughout the samples (Fig. 5). Since data for the July samples were obtained from pooled haemolymph samples, they were not included in the statistical analysis testing the seasonal effect. Nevertheless, the overall highest values of PDH (13%) were observed at the beginning of the sampling period (July 2000). On individually collected haemolymph samples from October 2000 till August 2001, this percentage showed significantly higher values in October, as compared to those measured after October (Table 1,  $P < 0.0001$ ). Comparing the sites, the PDH was significantly higher in clams from Chausey as compared to those of other sites in October 2000 and August 2001 (Fig. 5,  $P < 0.001$ ). The clams from the Bay of Brest showed significantly higher PDH in February 2001, which corresponded to the occurrence of mass mortalities at this site (Fig. 5,  $P < 0.001$ ). No differences of PDH between the three remaining sites were measured in April (Fig. 5,  $P = 0.07$ ).

Haemocyte size showed a seasonal pattern with haemocytes being larger during the winter and smaller during the summer (Table 1,  $P < 0.0001$ ). Haemocyte size showed significant differences between some sites (Fig. 6a,  $P < 0.05$ ). For example, clam haemocytes from clams in the Gulf of Morbihan were significantly smaller compared to those of clams in Chausey from February until August (Fig. 6b,  $P < 0.01$ ). No seasonal pattern was observed in haemocyte complexity (Table 1); however, this parameter showed significant differences between some sites (Fig. 6b,  $P < 0.05$ ). In October 2000, the haemocyte complexity was significantly higher in the clams seeded in Brittany (Bay of Brest and Gulf of Morbihan) than those in the other sites (Marennes and Chausey) (Fig. 6b,  $P < 0.0001$ ). From February to August 2001, the clams from the site of Marennes always exhibited significantly higher haemocyte complexity than those of clams cultivated in Chausey and in the Gulf of Morbihan (Fig. 6b,  $P < 0.05$ ). A seasonal pattern of protein content of clams was observed with high values in summer–autumn and low values in winter–spring at all sites (Table 1,  $P < 0.0001$ ). The protein content of the haemolymph showed significant differences between sites (Table 3,  $P < 0.001$ ). In October, the level of haemolymph proteins was significantly higher (1.1–1.2 mg mL<sup>-1</sup>) for clams in Chausey and Marennes compared to clams from the Brittany sites (0.5–0.6 mg mL<sup>-1</sup>) (Table 3,  $P < 0.0001$ ). In February, the protein content in clams from the Bay of Brest was found to be very low (0.2 mg mL<sup>-1</sup>) (Table 3,  $P < 0.001$ ). Haemolymph protein content in clams from

Chausey was significantly higher than those in clams from Gulf of Morbihan and Marennes in April (Table 3,  $P < 0.001$ ). The site effect was particularly marked in August with 1.5 mg mL<sup>-1</sup> in clams from Chausey, 0.8 mg mL<sup>-1</sup> in clams from Marennes, and 0.6 mg mL<sup>-1</sup> in clams from Gulf of Morbihan (Table 3,  $P < 0.0001$ ). Overall, sites can be classified according to their haemolymph protein content of clams in the following descending order: Chausey, Marennes, Gulf of Morbihan, and Bay of Brest.

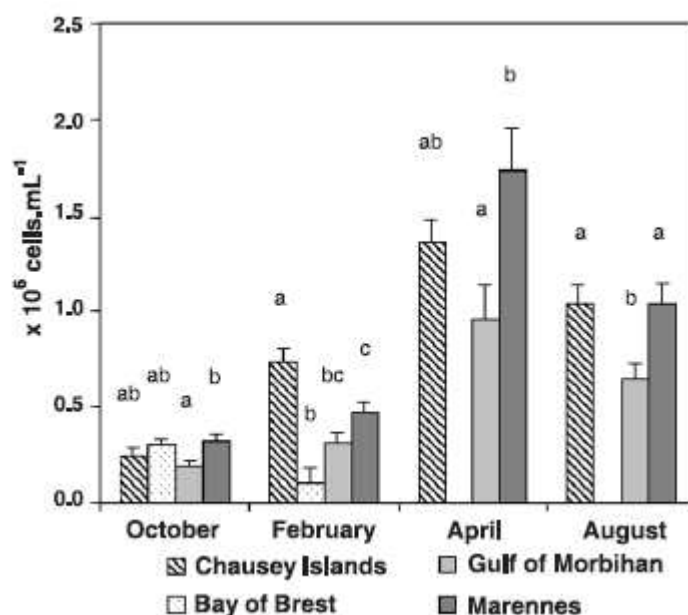


Fig. 4. Total haemocyte count (THC) expressed as  $10^6$  cells/mL (mean, S.E.,  $n = 14-20$  for October samples,  $n = 19-21$  for February, April and August samples). Lettering indicates the significant differences between sites for each sampling period (using Kruskal-Wallis test).

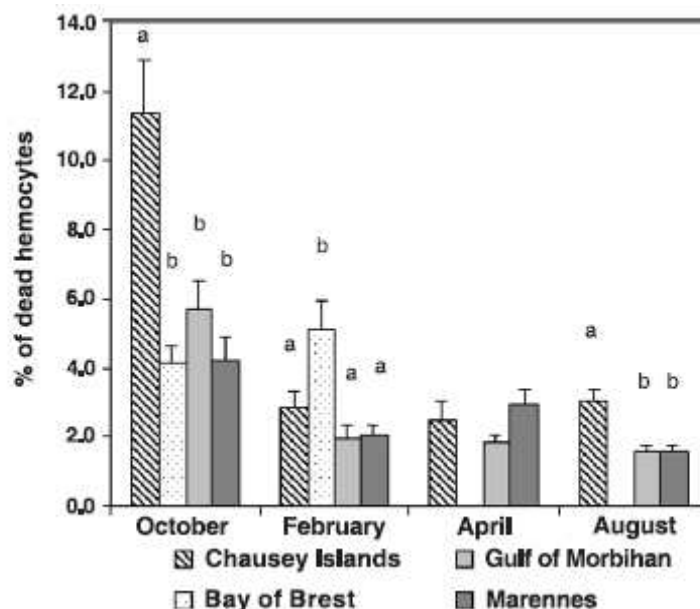


Fig. 5. Haemocyte mortality expressed as percentage of total cells (mean, S.E.,  $n = 14-20$  for October samples,  $n = 19-21$  for February, April and August samples). Lettering indicates the significant differences between sites for each sampling period (using Kruskal-Wallis test).



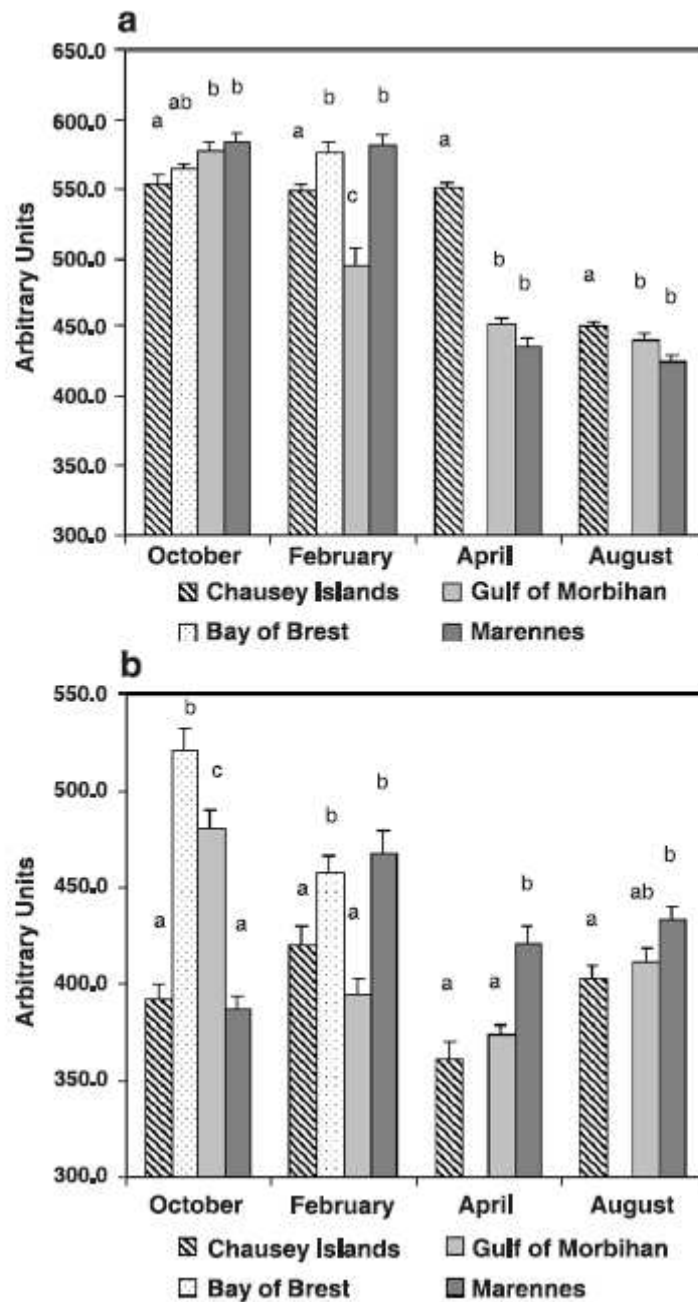


Fig. 6. (a) Haemocyte size (FSC) expressed as arbitrary units (mean, S.E.,  $n = 14-20$  for October samples,  $n = 19-21$  for February, April and August samples). Lettering indicates the significant differences between sites for each sampling period (using Kruskal–Wallis test). (b) Haemocyte complexity (SSC) expressed as arbitrary units (mean, S.E.,  $n = 14-20$  for October samples,  $n = 19-21$  for February, April and August samples). Lettering indicates the significant differences between sites for each sampling period (using Kruskal–Wallis test).

Lysozyme content decreased dramatically from October to February in three of four sites: at Chausey, Gulf of Morbihan and Bay of Brest (Fig. 7). Combining all sites, the overall decrease after autumn was statistically significant (Table 1,  $P < 0.001$ ). The location of rearing sites appeared to be of great importance for the lysozyme content. Clams reared in Marennnes had a significantly higher lysozyme content compared to other sites from February until August (Fig. 7,  $P < 0.0001$ ). Clam lysozyme content in sites can be classified in the following descending order: Marennnes, Chausey, Gulf of Morbihan and Bay of Brest.

Table 3 : Site effect on the protein content of the hemolymph (mg mL<sup>-1</sup>) during the field survey Chausey Bay of Brest Gulf of Morbihan Marennes.

	Chausey		Bay of Brest		Gulf of Morbihan		Marennes						
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	P value				
October	1.11 (n=19)	0.05	a	0.52 (n=14)	0.02	b	0.61 (n=19)	0.05	b	1.21 (n=21)	0.04	a	<0.0001
February	0.65 (n=19)	0.04	a	0.22 (n=17)	0.02	b	0.68 (n=20)	0.01	a	0.36 (n=19)	0.03	c	<0.0001
April	0.74 (n=20)	0.03	a	dead			0.56 (n=20)	0.02	b	0.57 (n=20)	0.08	b	<0.001
August	1.49 (n=15)	0.09	c	dead			0.57 (n=20)	0.07	a	0.82 (n=20)	0.05	b	<0.0001

Results were expressed as mean and standard error (S.E.). Lettering indicates the significant differences between groups of each parameter such that similar letters signify no significant difference between other values of the same parameter. Statistical analysis were performed using Kruskal–Wallis test (K–W).

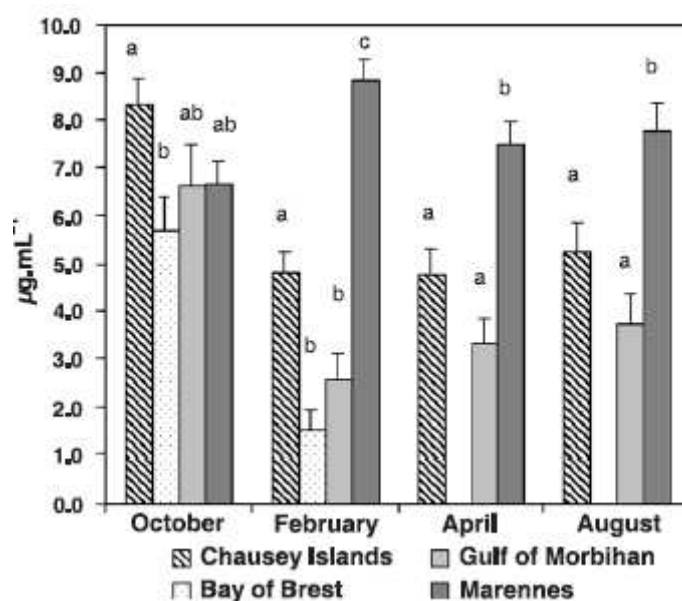


Fig. 7. Haemolymph lysozyme activity expressed as  $\mu\text{g mL}^{-1}$  (mean, S.E., n = 7–20). Lettering indicates the significant differences between sites for each sampling period (using Kruskal–Wallis test).

Linear regression analysis was performed on the mean or, where possible, on individual values of the physiological and immunological parameters. Linear regressions are presented in Table 4 for the correlation between immunological parameters and for the correlation between physiological and immunological parameters. Based on the established correlations, haemocyte size was negatively related to THC and positively to haemocyte mortality ( $P < 0.05$ ). Significant relationships between physiological and immunological parameters were also observed. Total haemocyte count, and protein and lysozyme contents were positively correlated to the condition index ( $P < 0.05$ ,  $P < 0.05$ , and  $P < 0.1$ , respectively). THC was also positively related to the dry weight of clams ( $P < 0.05$ ).

#### 4. Discussion

In all sites, we observed a seasonal pattern for the condition index (CI) with the highest values in summer and the lowest values in winter. Most of the growth (wet weight and dry weight increases) was observed in spring and summer. These observations were in agreement with those of Paillard (1992) on the CI of *V. philippinarum* and Laruelle et al. (1994) on the annual reproductive cycles of *V. philippinarum* and *V. decussatus* in Brittany. In the Bay of Brest, Laruelle et al. (1994) described an increase in CI from March until mid-August associated with gametogenesis and with two partial spawnings. The major decrease, which occurred in late August and early September, was attributed to complete spawning. In the Gulf of Morbihan, the CI increased dramatically from March until early June and then showed three major decreases corresponding to three complete spawnings in June, July and September. Goulletquer (1989) reported two major spawnings in Marennes, one at the end of spring and another at the end of the summer. Laruelle et al. (1994) clearly demonstrated that the CI variation

was closely related to maturation stages of *V. philippinarum* which are well known to be controlled by temperature and food availability (Mann, 1979; Laing and Lopez-Alvarado, 1994; Sbrenna and Campioni, 1994).

Table 4 : Correlation between physiological and immunological parameters.

	Correlation coefficient	$R^2$	P value
<i>Within immunological parameters</i>			
Haemocyte size and log (THC <sup>a</sup> )	–	0.39	<0.05
Haemocyte mortality and log (THC <sup>a</sup> )	–	0.30	<0.05
Haemocyte size and H. mortality	+	0.29	<0.05
<i>Between physiological and immunological parameters</i>			
Condition index and protein content	+	0.35	<0.05
Dry weight and log (THC <sup>a</sup> )	+	0.34	<0.05
Condition index and log (THC <sup>a</sup> )	+	0.31	<0.05
Condition index and lysozyme content	+	0.26	<0.1

<sup>a</sup> Total haemocyte count.

A site effect was especially clear for total weight and dry weight. The fastest growth was obtained in Marennes with a wet weight increase of 20 g in 1 year, which is an excellent performance (Flassch and Leborgne, 1992). In less than 1 year and without any evidence of BRD, these clams were marketable. The better growth obtained in Marennes could be explained by the higher temperature in this site but also a higher food availability. Indeed, Marennes ponds are known to have a tremendously higher primary production compared to estuarine or oceanic culture sites, especially during spring and fall (Robert et al., 1982; Goulletquer et al., 1988); however, this primary production was observed to decrease because of lack of nitrogen supply and due to intense filter feeding by bivalves during the summer in Marennes–Oléron area (Robert et al., 1982; Goulletquer et al., 1988). This is proposed as an explanation of the lower reproductive effort, measured according to Lucas et al. (1978), in Marennes (Goulletquer, 1989) compared to the Bay of Brest or the Gulf of Morbihan (Laruelle, 1999). No literature is available on clams reared in Chausey Islands, venerid culture started less than 5 years ago in this area. A reduced reproductive effort, in agreement with the maintenance of CI below 10 (reduced germinal growth) in the present study during the summer in Marennes ponds, may also favour the overall higher growth performance (somatic growth) obtained in Marennes ponds. In both Brittany sites, clams experienced massive mortalities during the winter in the Bay of Brest and during the summer in the Gulf of Morbihan. Winter mortalities in clams were frequently observed on the north coast of Brittany ranging from 10% to 80% (Goulletquer, 1989; Paillard, 1992). Winter mortalities of clams in the rearing site at the Bay of Brest have also been described by Calvez and Guillou (1998) who proposed that a reduced food supply was a weakening factor. Paillard (1992) suggested that oxygen depletion and high sulfur concentrations in sediment, due to algal deposition after winter storms, was associated with reduced clam CI and glycogen reserves and thus can contribute to winter mortality. Massive mortalities and low CI observed in Gulf of Morbihan during the last summer of the experiment could not be clearly explained but was suspected to be associated with intense algal fouling on the net kept on the experimental plot and/or oxygen depletion in the mud in this site.

During this field survey, some immunological parameters were clearly affected by both location of rearing sites and seasonal variations. Seasonal effects were only demonstrated for THC, haemocyte size and protein content in haemolymph. High values of THC and protein content were observed in spring–summer and low values in autumn–winter. In contrast, haemocyte size was high in autumn–winter and low in spring–summer. Thus, haemocyte size was related to low total haemocyte count (THC), a relationship that can be explained by variation of the cell division rate. When cell division occurred, the size of resulting cells is speculated to be smaller than the size of mature cells. Carballal et al. (1998) described a seasonal pattern with THC in mussels *Mytilus galloprovincialis* being positively correlated with water temperature. For the oyster *Crassostrea virginica*, THC was also observed to be high during summer and low during winter (Chu et al., 1995; Volety et al., 1999).

Protein content of clams followed a seasonal pattern with high values in summer–autumn and low values in winter–spring at all sites. Haemolymph protein content has already been demonstrated to depend on the season although an inverse trend with the highest values in winter was found for the oyster *C. virginica* (Fisher and Newell, 1986; Chu and La Peyre, 1989; Fisher et al., 1996). This difference can be traced to the ranges of protein content which appeared to be species-specific, 2.5–8.0 mg mL<sup>-1</sup> for *C. virginica* (Fisher et al., 1996) and 0.2–1.4 mg mL<sup>-1</sup> for *V. philippinarum* (this study). The latter values are in agreement with the values found by Oubella et al. (1994) and Allam et al. (2000a) which were around 0.3 mg mL<sup>-1</sup> in haemolymph of Manila clams. Similar protein contents, ranging from 0.4 to 1.0 mg mL<sup>-1</sup>, were measured for *V. decussatus* (Ordas et al. 2000).

The percentage of dead haemocytes was observed to vary greatly during the field survey. However, the variations were difficult to associate with a seasonal pattern. Indeed, the small size of the animals and resulting

difficulty in obtaining the haemolymph sample may explain the high percentage of dead haemocytes observed at the beginning of the experiment (July). However, the significantly higher percentage of dead haemocytes measured in October, as compared to others measured after October, was associated with the lowest THC and to a decrease of CI during this period. As mentioned earlier, this decrease not only reflects the completion of the spawning period, which occurred by the end of the summer, but also the decreasing food availability during the fall (Goulletquer, 1989; Laruelle, 1999). Interestingly, a higher percentage of dead haemocytes was observed in the Bay of Brest in February when animals were experiencing massive mortalities. Paillard et al. (1996) indicated that a high percentage of dead haemocytes may be a good indicator of the physiological status especially before or during mortality events. No seasonal pattern was observed for the lysozyme content although higher lysozyme contents in clams were observed in October for the Normandy and Brittany sites. In *C. virginica*, several studies found a seasonal pattern with higher lysozyme content during winter and lower values during the summer (Chu and La Peyre, 1989; Chu et al., 1995; Volety et al., 1999); however, Fisher et al. (1996) observed higher lysozyme levels in July and lower levels during fall and winter. In controlled conditions, Chu and La Peyre (1993) observed that after 20 days of temperature acclimatisation, lysozyme content was higher at 10 and 15 °C than at 20 and 25 °C. In the present study, the lysozyme content was correlated well with the location of rearing sites and sites could be ranked according to their lysozyme content, in the following descending order: Marennes, Chausey, Gulf of Morbihan and Bay of Brest. The greater food availability in Marennes ponds (as mentioned above for the growth performance) may be an explanation of the maintenance of a high lysozyme content in clams from this site. Indeed, the bacteriolytic enzyme lysozyme is believed to participate in the digestion of bacteria which can form a significant part of bivalve diet (McHenery et al., 1986).

Although some relationships have been established experimentally between BRD development and physiological and immunological modifications (Plana et al., 1996; Oubella et al., 1993, 1994, 1996; Allam, 1998; Allam et al., 2000a,b), their significance in the field could not be assessed during the present study because BRD prevalence was very low. The natural population of clams close to the experimental clams in the Gulf of Morbihan had a much higher prevalence of BRD, suggests that hatchery seed used in this experiment may be BRD resistant. Moreover, the same hatchery stocks from Marennes were experimentally challenged with *V. tapetis* after various salinity exposures and showed also low prevalence of BRD development (Reid et al., in press). Though both experiments are in agreement with the hypothesis of BRD resistance, this deserves further investigations and new experimental in vivo challenges must be performed in the laboratory. Although this study cannot be used to assess which environmental factors have the greatest impact on animal health, it provides valuable information on the range of variation of these parameters in the field. However, this study can be useful to generate hypotheses and thus design experiments aiming to discriminate the impact of individual environmental factors (physico-chemical, pollutants, food availability and quality, parasitism) as proposed by Fisher et al. (2000). It can also help construct numerical models exploring growth performance and disease susceptibility. Interestingly, total haemocyte count and protein and lysozyme concentrations were positively correlated to the condition index. Moreover, the winter mortality event in Bay of Brest has been associated with a depressed immunological status (high percentage of dead haemocytes, low THC, low lysozyme activity). Thus, these haematological parameters are proposed as pertinent tools to assess physiological status in association with other indices. Haematological parameters can also be considered as nondestructive since repeated haemolymph sampling in adductor muscle had no measurable effect on survival of the clam *V. philippinarum* (Paillard and Ford, 2002). Advancements in the use of tools for assessing physiological and immunological status can help clam farmers to locate the best sites for favourable development of venerid culture. Regarding the low BRD prevalence in seeded clams, further investigations are also needed to verify if the hatchery-spat stocks are disease resistant.

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